LuxS-Based Signaling Affects *Streptococcus mutans* Biofilm Formation

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*Streptococcus mutans* is implicated as a major etiological agent in human dental caries, and one of the important virulence properties of this organism is its ability to form biofilms (dental plaque) on tooth surfaces. We examined the role of autoinducer-2 (AI-2) on *S. mutans* biofilm formation by constructing a GS-5 luxS-null mutant. Biofilm formation by the luxS mutant in 0.5% sucrose defined medium was found to be markedly attenuated compared to the wild type. Scanning electron microscopy also revealed that biofilms of the luxS mutant formed larger clumps in sucrose medium compared to the parental strain. Therefore, the expression of glucosyltransferase genes was examined and the *gtfB* and *gtfC* genes, but not the *gtfD* gene, in the luxS mutant were upregulated in the mid-log growth phase. Furthermore, we developed a novel two-compartment system to monitor AI-2 production by oral streptococci and periodontopathic bacteria. The biofilm defect of the luxS mutant was complemented by strains of *S. gordonii*, *S. sobrinus*, and *S. anginosus*; however, it was not complemented by *S. oralis*, *S. salivarius*, or *S. sanguinis*. Biofilm formation by the luxS mutant was also complemented by *Porphyromonas gingivalis* 381 and *Actinobacillus actinomycetemcomitans* Y4 but not by a *P. gingivalis* luxS mutant. These results suggest that the regulation of the glucosyltransferase genes required for sucrose-dependent biofilm formation is regulated by AI-2. Furthermore, these results provide further confirmation of previous proposals that quorum sensing via AI-2 may play a significant role in oral biofilm formation.

Quorum sensing (QS) is a process whereby bacteria communicate with one another by means of the secretion of chemical signal molecules called autoinducers (AIs) (3, 4, 35, 38). In the bioluminescent gram-negative marine bacterium *Vibrio harveyi*, two distinct AIs, AI-1 (6, 9) and AI-2, regulate light emission (36). LuxS is an enzyme involved in the catabolism of S-adenosylmethionine and converts ribose homocysteine into homocysteine and 4,5-dihydroxy-2,3-pentanedione, the precursor of AI-2 (6, 46, 51). This system has been referred to as an interspecies quorum system and may operate as a universal quorum system for many bacteria possessing the characteristic luxS gene (5). The luxS gene is highly conserved across a diverse range of gram-negative and gram-positive bacterial species, and AI-2 is produced by many of these species. QS enables a population of bacteria to regulate gene expression including expression of virulence factors (21, 39), competence for genetic transformation (1, 28, 37), conjugal DNA transfer (20, 52, 56), and the production of antibiotics and secondary metabolites (31, 49), as well as biofilm formation (13). However, more recent investigations have also indicated that AI-2 production is regulated at the level of LuxS substrate availability and not at the level of luxS expression. Consequently, AI-2-dependent signaling can also reflect the metabolic state of the cell and not necessarily cell density (7).

Biofilms are sessile communities of microorganisms attached to a surface (12, 41, 53). It is clear that microorganisms undergo profound changes during their transition from planktonic organisms to cells that are part of a complex, surface-attached community. These changes are reflected in the new phenotypic characteristics developed by biofilm bacteria and occur in response to a variety of environmental signals (22, 40). Formation of these sessile communities and their inherent resistance to antimicrobial agents are important factors in many persistent and chronic bacterial infections (13, 30).

*Streptococcus mutans* is the principal causative agent of dental caries in humans and its ability to adhere to the tooth surface is paramount for the progression of disease (17, 29). One of the important virulence properties of these organisms is their ability to form biofilms known as dental plaque on tooth surfaces (27). Dental plaque, one of the best-studied biofilms, is a complex community comprising more than 500 bacterial species (23, 24, 43). The early colonizers of the enamel surfaces are predominantly streptococci, which form mixed-species microcolonies during early plaque development (25). To initiate heterogeneous bacterial interactions, diffusible signals may play important roles resulting in dental plaque formation (33).

Among the potential signaling molecules, the competence-stimulating peptides (CSP), approximately 21-mer cationic oligopeptides, regulate the competence pathways of streptococci (1), as well as *S. mutans* biofilm formation (28, 54). Recently, another diffusible signal molecule, AI-2, was identified in several oral bacteria (8, 11, 14, 24, 34). In the present study, we describe the biofilm phenotype of a *S. mutans* GS-5 luxS mutant and how AI-2 molecules affect biofilm formation of these organisms. In addition, we have developed a novel assay system for monitoring AI-2 levels using complementation of the *S. mutans* luxS mutant. Using this assay system, we show that some, but not all, oral streptococci, as well as selected gram-negative oral bacteria, complement the luxS mutation in *S. mutans*. These results provide a molecular explanation for the dependence of *S. mutans* sucrose-dependent biofilm formation on AI-2 and further suggest that LuxS-dependent signaling...
may mediate intra- and interspecies responses among bacteria in oral biofilms as recently proposed (33).

**MATERIALS AND METHODS**

**Bacterial strains and culture conditions.** Bacterial strains used in this study are listed in Table 1. All streptococcal species were grown anaerobically at 37°C in brain heart infusion (BHI; Difco Laboratories, Detroit, Mich.) broth or chemically defined medium (CDM) (54, 55) supplemented with appropriate carbon sources. Transformants of S. mutans were selected after growth on mitis salivarius agar (Difco Laboratories) plates supplemented with erythromycin (10 \( \mu \)g/ml) and supplemented with yeast extract, 0.5% NaCl medium at 37°C. For V. harveyi strains 381 and CW221 (luxS mutant), 381::pVA2198; luxS were cultured at 30°C in Luria-marine (LM) medium (20 g of NaCl, 10 g of Bacto tryptone, and 5 g of yeast extract/liter).

**DNA manipulations.** DNA isolation, endonuclease restriction, ligation, and transformation of competent E. coli cells were carried out as previously described (45). Transformation of S. mutans was accomplished by procedures routinely carried out in this laboratory (44).

**Construction of the luxS mutant.** The open reading frame for the luxS gene and the flanking regions were identified in the S. mutans UA159 database from the University of Oklahoma Advanced Center for Genome Technology (http://genome.ou.edu.smutans.html). The luxS-null mutant was constructed by allelic exchange via insertion of an erythromycin resistance (Ery\(^r\)) determinant into the gene. The plasmid used for disruption of the luxS gene was prepared as follows. The PCR fragments of the upstream and downstream regions of luxS were amplified with the primers luxSUF2883(Sma) and luxSUR3884(Bam), respectively, using chromosomal DNA from S. mutans UA159 and the flanking regions were identified in the S. mutans UA159 database from the University of Oklahoma Advanced Center for Genome Technology (http://genome.ou.edu.smutans.html). The luxS-null mutant was constructed by allelic exchange via insertion of an erythromycin resistance (Ery\(^r\)) determinant into the gene. The plasmid used for disruption of the luxS gene was prepared as follows. The PCR fragments of the upstream and downstream regions of luxS were amplified with the primers luxSUF2883(Sma) and luxSUR3884(Bam), respectively, using chromosomal DNA from S. mutans UA159 and the flanking regions were identified in the S. mutans UA159 database from the University of Oklahoma Advanced Center for Genome Technology (http://genome.ou.edu.smutans.html). The luxS-null mutant was constructed by allelic exchange via insertion of an erythromycin resistance (Ery\(^r\)) determinant into the gene. The plasmid used for disruption of the luxS gene was prepared as follows. The PCR fragments of the upstream and downstream regions of luxS were amplified with the primers luxSUF2883(Sma) and luxSUR3884(Bam), respectively, using chromosomal DNA from S. mutans UA159 and the flanking regions were identified in the S. mutans UA159 database from the University of Oklahoma Advanced Center for Genome Technology (http://genome.ou.edu.smutans.html). The luxS-null mutant was constructed by allelic exchange via insertion of an erythromycin resistance (Ery\(^r\)) determinant into the gene. The plasmid used for disruption of the luxS gene was prepared as follows. The PCR fragments of the upstream and downstream regions of luxS were amplified with the primers luxSUF2883(Sma) and luxSUR3884(Bam), respectively, using chromosomal DNA from S. mutans UA159 and the flanking regions were identified in the S. mutans UA159 database from the University of Oklahoma Advanced Center for Genome Technology (http://genome.ou.edu.smutans.html). The luxS-null mutant was constructed by allelic exchange via insertion of an erythromycin resistance (Ery\(^r\)) determinant into the gene.

**Assay for in vitro biofilms.** (i) Quantification of biofilm formation. Biofilm formation was quantified as previously described (54). Flat-bottom polystyrene microtiter plates (enzyme immunoassay-radioimmunoassay plates, 96-well Easy Wash: Corning, Inc., Corning, N.Y.) containing 100 \( \mu \)l of CDM per well were inoculated with S. mutans GS-5 and its luxS mutant (1.7 \( \times \) 10^5 CFU per well) after 24-h growth in BHI. The bacteria grown in BHI were centrifuged and washed with CDM and then suspended with the same amount of CDM.

**TABLE 1. Bacterial strains and plasmids**

<table>
<thead>
<tr>
<th>Strains</th>
<th>Relevant characteristics or distributions</th>
<th>Source or reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>S. mutans GS-5</td>
<td>Ery(^r); Kan(^r); serotype c human isolate</td>
<td>SUNYaB*</td>
</tr>
<tr>
<td>S. mutans ΔluxS</td>
<td>Ery(^r); GS-5:pAYLS1101; luxS</td>
<td>This study</td>
</tr>
<tr>
<td>P. gingivalis 381</td>
<td>Type strain</td>
<td>KU^b</td>
</tr>
<tr>
<td>P. gingivalis CW221</td>
<td>Ery(^r); 381::pVA2198; luxS</td>
<td>Wen and Kuramitsu (SUNYaB)</td>
</tr>
<tr>
<td>A. actinomycetemcomitans Y4</td>
<td>Serotype b human isolate</td>
<td>KU</td>
</tr>
<tr>
<td>S. sobrinus MT8145</td>
<td>Mutans group oral streptococci, serotype d</td>
<td>KU</td>
</tr>
<tr>
<td>S. gordonii DL1</td>
<td>Mitis-sanguinis group oral streptococci</td>
<td>KU</td>
</tr>
<tr>
<td>S. oralis ATCC10557</td>
<td>Mitis-sanguinis group oral streptococci</td>
<td>ATCC^c</td>
</tr>
<tr>
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<td>Mitis-sanguinis group oral streptococci</td>
<td>ATCC</td>
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<td>S. salivarius HT9R</td>
<td>Salivarius group oral streptococci</td>
<td>KU</td>
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<td>S. anginosus FW73</td>
<td>Aniginosus group oral streptococci</td>
<td>KU</td>
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<tr>
<td>V. harveyi BB120</td>
<td>Wild type</td>
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<tr>
<td>V. harveyi BB170</td>
<td>luxS::Tn5; AI-1 sensor(^+); AI-2 sensor(^+), reporter strain</td>
<td>51</td>
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<td>Cloning host</td>
<td>Invitrogen</td>
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<tr>
<td>pBluescript II SK(+)</td>
<td>Amp(^r); cloning vector</td>
<td>Stratagene</td>
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<tr>
<td>pResEmMCS10</td>
<td>Ery(^r); integration vector</td>
<td>47</td>
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<tr>
<td>pAYLS1101</td>
<td>Amp(^r); Ery(^r); pBluescript II SK(+) bearing luxS upstream and downstream regions and Ery(^r) gene</td>
<td>This study</td>
</tr>
</tbody>
</table>

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*a SUNYaB, the culture collection in Department of Oral Biology, State University of New York, Buffalo, N.Y.*

*b KU, the culture collection in Department of Preventive Dentistry, Kyushu University, Fukuoka, Japan.*

*c ATCC, American Type Culture Collection, Manassas, Va.*
Table 2. Oligonucleotide primers and probes

<table>
<thead>
<tr>
<th>Primer or probe</th>
<th>Nucleotide sequence</th>
<th>Gene</th>
<th>Size (bp)</th>
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<tr>
<td>luxSUF2883(Sma)</td>
<td>5′-CCC CCC CCC GGG TCT TCA ATT CGA GCA GGA-3′</td>
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<td>luxSUR3884(Bam)</td>
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<td>luxSDF4347(Bam)</td>
<td>5′-CCC CCC GGA TCC GTG ATC TAG TGT AAA AT-3′</td>
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<tr>
<td>luxSDR5396(Xba)</td>
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<td>gtfB-F241</td>
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<tr>
<td>gtfB-R539</td>
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<td>gtfC</td>
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<td>recA-R515R</td>
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<td>85</td>
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<tr>
<td>luxSDF4347(Bam)</td>
<td>5′-FAM-ATG TTA TTA ATG ATA GCA ATG C-NFQ-MGB-3′</td>
<td>luxS</td>
<td>1,002</td>
</tr>
<tr>
<td>luxSUR3884(Bam)</td>
<td>5′-FAM-AAC CCA AAT CCA GCA ACA AGC AGT TCT G-TAMRA-3′</td>
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<td>recA-F449</td>
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<td>recA-R515R</td>
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<td>recA</td>
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</table>

* Endonuclease recognition sequences are underlined.
* FAM, 6-carboxyfluorescein; NFQ, nonfluorescent quencher; MGB, minor groove binder; TAMRA, 6-carboxytetr methylrhodamine.

cultures were diluted to 1:100 and 100 μl of bacterial solution was added to each well. After 48 h of incubation at 37°C, 25 μl of 1% (wt/vol) crystal violet (CV) solution was added to each well. After 15 min, the wells were rinsed three times with 200 μl of distilled water and air dried. The CV on the abiotic surface was determined. Growth was determined by measuring the turbidities (OD570) of parallel wells after resuspension of the sessile organisms with the planktonic cells.

(ii) Two-compartment system. Each well of a 96-well polystyrene plate was separated into two compartments by using 0.2-μm-pore-size Anapore membranes (Nunc tissue culture inserts, 8-well strip; Nalge Nunc International). The 5-by-5-mm polystyrene tips and the membranes were separated into two compartments by using 0.2-μm-pore-size Anapore membranes (Nunc tissue culture inserts, 8-well strip; Nalge Nunc International, Naperville, Ill.). The S. mutans luxS mutant was inoculated into the lower compartment, and other streptococci or gram-negative bacteria were inoculated into the upper compartment (Fig. 1). After coinoculation of the bacteria in both upper and lower compartments, the membrane strips were removed, and the amount of parallel wells after resuspension of the sessile organisms with the planktonic cells.

SEM. Biofilms formed on polystyrene surfaces were examined by scanning electron microscopy (SEM) to verify the quantitative results observed. Biofilms were anaerobically inoculated on 5-by-5-mm polystyrene tips in six-well polystyrene dishes. For the bacterial complementation analysis, the two-compartment assay was carried out by using six-well polystyrene dishes (Corning, Inc.) and 0.2-μm-pore-size Anapore membranes (Nunc 25-mm tissue culture inserts; Nalge Nunc International). The 5-by-5-mm polystyrene tips and the S. mutans luxS mutant were inoculated into the lower compartment, and the indicated streptococci or gram-negative bacteria were added into the upper compartment. After inoculation, biofilms on the polystyrene tips were washed once in distilled water, fixed with formaldehyde, and incubated at 20°C overnight. After dehydration through a graded series of ethanol, the polystyrene tips were air dried and sputter coated with gold. Samples were then examined at 500 to 7,500 magnification by using SEM (JEOL JSM-5400LV; JEOL Techniques, Ltd., Tokyo, Japan).

Al-2 bioassay for oral streptococci. S. mutans strains and other streptococci were cultured in BHI (Difco) broth to stationary phase. The stationary-phase culture was then inoculated into CDM supplemented with 0.5% sucrose until OD570 reached 0.5. The culture supernatant was centrifuged and passed through a 0.2-μm-pore-size filter to remove cells. Al-2 bioassays with V. harveyi BB170 were performed basically as previously described (51). Briefly, V. harveyi BB170 was grown overnight at 30°C in LM medium. The culture was diluted 1:5,000 in fresh Al bioassay medium (AB medium: 0.3 M NaCl, 0.05 M MgSO4, 0.2% Casamino Acids, 1.0% potassium phosphate, 1.0 mM L-arginine, 2% glycerol, 0.01 μg of riboflavin/ml, 1 μg of thiamine HCl/ml, and 90 μl of the diluted culture supernatant was added at a 10% (vol/vol) final concentration into wells of 96-well microtiter plates. Positive control wells contained 10 μl of cell-free conditioned medium from V. harveyi BB170, whereas negative control wells contained 10 μl of sterile AB medium. Luminescence was measured every hour with a Luminometer (Fluoroscan Ascent Flto; Thermo Labsystems, Vantaa, Finland).

Real-time quantitative RT-PCR. Oligonucleotide primers and probes for the gf genes, designed by using Primer Express 1.5 software (Applied Biosystems, Foster City, Calif.), are listed in Table 2. The primers for recA, used as an internal control, were also designed by using Primer Express 1.5 software. The fluorescent probes were labeled with a reporter dye (6-carboxyfluorescein) covalently attached at the 5′ end, and a quencher dye (6-carboxytetramethylrhodamine) covalently attached at the 3′ end. Total RNA was isolated from S. mutans GS-5 and the luxS mutant by using TRIzol Reagent (Gibco-BRL, Rockville, Md.) according to the manufacturer’s instructions. Single-stranded cDNA was then synthesized in a reaction mixture containing 1.25 U of Multiscribe reverse transcriptase/μl, 0.4 U of RNase inhibitor/μl, 500 mM concentrations of each dNTP, 200 mM concentrations of deoxynucleoside triphosphate, 0.2 mM MgCl2 (TaqMan reverse transcription reagents; Applied Biosystems), and 1.0 μg of total RNA from each phase of culture at 48°C for 30 min. To check for DNA contamination, purified total RNA without reverse transcriptase served as a negative control. The resulting cDNA and negative control were amplified by using the TaqMan Universal PCR Master Mix (Applied Biosystems), which contained deoxynucleoside triphosphates with dUTP, AmpliTaq Gold DNA polymerase, Amperase UNG, optimized buffer, and a passive reference dye. For each PCR, a mixture containing template...
cDNA, 1× Master Mix, 200 nM concentrations of each forward and reverse primer, and 250 nM TaqMan probe was applied to a 96-well MicroAmp optical reaction plate with optical caps (Applied Biosystems). Amplification and detection of specific products were performed on the ABI Prism 7700 sequence detection system (PE Biosystems) with the following cycle profile: 1 cycle at 50°C for 2 min, 1 cycle at 95°C for 10 min, 60 cycles at 95°C for 15 s, and 60°C for 1 min. The critical threshold cycle (CT) was defined as the cycle at which the fluorescence becomes detectable above background and is inversely proportional to the logarithm of the initial number of template molecules. A standard curve was plotted for each primer-probe set with CT values obtained from amplification of known quantities of cDNA. To check the linearity of the detection system, a cDNA dilution series (1/10, 1/100, 1/1,000, and 1/10,000) was amplified with primer pairs and probes so that a correlation coefficient could be calculated from the standard curve displaying CT values. The standard curves were used to transform CT values to the relative number of cDNA molecules. The quantities of cDNA for gtfB, gtfC, and gtfD normalized to cDNA synthesized from recA were compared.

RESULTS

Nutrient factors affecting biofilm formation by the S. mutans luxS mutant. In order to analyze how the difference in the carbon source affects biofilm formation by the S. mutans luxS mutant, we examined glucose- and sucrose-dependent biofilm formation of this mutant. We found that biofilm formation by the luxS mutant in CDM supplemented with 0.5 or 1.0% glucose was not greatly attenuated compared to that of the parent strain, GS-5 (Fig. 2). However, biofilm formation by the luxS mutant in CDM supplemented with 0.5% sucrose was found to be markedly attenuated compared to the parent strain (Fig. 3A and B).

SEM analysis of wild-type and luxS mutant biofilms. In addition, to further assess the biofilm phenotype of the luxS mutant in medium supplemented with different carbon sources, we also used SEM analysis (Fig. 4). The luxS mutant showed no significant qualitative difference in the biofilm phenotypes compared to GS-5 in the media supplemented with 0.5% glucose (Fig. 4A and B). In contrast, the luxS mutant formed biofilms that markedly differed from GS-5 in morphology when grown with 0.5% sucrose (Fig. 4C and D). The luxS mutant exclusively formed very large clumps in medium supplemented with sucrose compared to the biofilms formed by parental GS-5 strain (Fig. 4C).

Quantification of glucosyltransferase gene transcription in the S. mutans luxS mutant. The increased sucrose-dependent colonization of hard surfaces by the luxS sessile cells could result from increased insoluble glucan synthesis catalyzed by the glucosyltransferases (Gtfs) of the mutant. In order to assess gtf gene expression in the S. mutans luxS mutant, real-time PCR assays were used for the quantification of the transcript levels of the gtfB, gtfC, and gtfD genes using an equal amount of total RNA from different growth stages in CDM supplemented with 0.5% sucrose. Initially, we evaluated the transcription levels of the recA gene in the luxS mutant and GS-5 in each culture phase. We observed no significant differences in the expression of the recA gene from samples in each culture phase (data not shown). The gtfBCD mRNA levels were almost the same in the early-log and late-log phases within each strain. However, the mRNA expression levels of the gtfB and gtfC genes, coding for Gtfs involved in insoluble glucan synthesis, in
the luxS mutant were almost 5.5- and 3-fold higher, respectively, compared to GS-5 in the mid-log phase (Fig. 5). On the other hand, gtfD, coding for a Gtf synthesizing soluble glucans, expression in the luxS mutant was slightly reduced in the mid-log phase compared to that of GS-5 (Fig. 5).

Complementation analysis of the luxS mutant by other oral bacteria by using the two-compartment assay system. In order to determine whether other oral bacteria can communicate with S. mutans via AI-2 signaling, we determined whether other bacteria could complement the luxS mutation of strain GS-5. For the bacterial complementation analysis, we used a novel two-compartment biofilm assay system (Fig. 1). Initially, we performed complementation analysis of the GS-5 luxS mutant by using oral streptococci in the CDM supplemented with 0.5% sucrose. Biofilm formation by the luxS mutant was restored to wild-type GS-5 levels in the presence of S. gordonii, S. sobrinus, and S. anginosus in the upper compartments (Fig. 6). However, no significant restoration was observed with S. oralis, S. salivarius, and S. sanguinis (Fig. 6). That this complementation was dependent upon AI-2 was demonstrated by the observation that GS-5, but not its luxS mutant, grown in the upper compartment complemented biofilm formation by the

FIG. 4. SEM images of S. mutans GS-5 and luxS mutant biofilms on polystyrene surfaces. Bacteria were incubated on polystyrene tips after 48 h of incubation. S. mutans GS-5 inoculated in CDM supplemented with 0.5% sucrose (A) and glucose (B). Both images were obtained at a ×1,000 magnification. luxS mutant inoculated in CDM supplemented with 0.5% sucrose (C) and glucose (D). Images were obtained at a ×1,000 magnification. SEM analysis of the S. mutans luxS mutant complemented by GS-5 (E). Bacteria were incubated in the 0.5% sucrose-CDM for 48 h. Images were obtained at ×1,000 magnification.
luxS mutant in the bottom compartment (Fig. 6). The phenotypic alteration of the S. mutans luxS mutant complemented by GS-5 was also analyzed by SEM. Compared to the large clumps of cells formed by the luxS mutant in the presence of sucrose (Fig. 4C), much smaller aggregates were detected when the mutant was complemented by the parental strain GS-5 (Fig. 4E), a result similar to that observed with the parental strain alone (Fig. 4A).

Furthermore, we performed complementation analysis with the gram-negative periodontopathic bacteria P. gingivalis and A. actinomycetemcomitans. Both organisms were recently demonstrated to secrete AI-2 (11, 14). P. gingivalis 381 and its luxS mutant were inoculated into the diluted TSB medium supplemented with 0.5% sucrose. Each strain was inoculated into the upper wells of the two-compartment system. Biofilm formation by the S. mutans luxS mutant was restored to parental levels when the luxS mutant was co inoculated with the P. gingivalis 381 parental strain. However, biofilm formation by the luxS mutant was not restored when co inoculated with the P. gingivalis luxS mutant (CW221) (Fig. 7A). In addition, biofilm formation by the S. mutans luxS mutant was restored to GS-5 levels when co cultured with A. actinomycetemcomitans Y4 (Fig. 7B).

AI-2 production in oral bacteria. To confirm that the various streptococci, P. gingivalis, and A. actinomycetemcomitans produce AI-2, we examined the AI-2 levels with V. harveyi BB170 (luxN::Tn5, AI-1 sensor –, AI-2 sensor +) as a reporter strain. Cell-free supernatants of the bacteria were added to V. harveyi BB170, and the luminescence induced by the culture supernatant of these oral bacteria was measured. The luminescence level of BB170 induced by supernatant from S. mutans GS-5 was ca. 8% of that stimulated by V. harveyi BB120 (Table 3). In contrast, the luminescence of BB170 was significantly decreased by supernatants prepared from the GS-5 luxS mutant compared to GS-5 (Table 3). The enhanced luminescence of BB170 was equivalent to GS-5 when stimulated by S. gordonii DL1 or S. sobrinus MT8145, S. salivarius HT9R and S. anginosus FW73 stimulated the luminescence of BB170 approximately twofold higher compared to S. mutans GS-5. However, the luminescence of BB170 strain was decreased by S. oralis ATCC 10557 and S. sanguinis ATCC 10556 compared to GS-5 (Table 3).

DISCUSSION

AI-2 is a novel bacterial signal produced by both gram-negative and gram-positive genera, and the LuxS-dependent QS circuit, originally identified in V. harveyi, was identified in many gram-negative and gram-positive bacteria (6). A recent study of AI-2 secretion by periodontal bacteria showed that P. gingivalis, Prevotella intermedia, Fusobacterium nucleatum, and A. actinomycetemcomitans secrete AI-2-like signals that can induce luminescence in V. harveyi (11, 14, 15). LuxS-based signaling is thought to represent an important means of intergeneric communication, especially in biofilms (32). Furthermore, individual species regulate different aspects of metabolism and virulence factor expression in response to AI-2. Recently, the luxS gene has also been isolated from P. gingivalis and appears to be important for regulating aspects of iron acquisition by this organism (11).

The nutrient content of the medium was found to regulate the development of biofilms in several organisms (10, 16, 42). For cariogenic dental pathogens, identification of the luxS gene for S. mutans GS-5 was recently reported (34). Therefore, we initially examined the effects of carbohydrates on biofilm formation by the S. mutans luxS mutant and the parental strain. Biofilm formation by these strains was equivalent in glucose-CDM. However, there was a marked difference in sucrose-mediated biofilm formation between these strains. Therefore, we hypothesized that the extracellular glucan synthesis genes are involved in luxS regulation of S. mutans sucrose-dependent biofilm formation.

For the confirmation of this hypothesis, we compared biofilm formation by these strains by using SEM analysis. In CDM supplemented with 0.5% glucose, the luxS mutant and GS-5 parental strain did not exhibit phenotypic differences. However, phenotypic differences between the luxS mutant and GS-5 were apparent in biofilms grown in CDM supplemented with 0.5% sucrose. In the sucrose-CDM, the luxS mutant formed many large clumps of cells on the surface of polysty-
rene (Fig. 4C). A recent report by Merritt et al. also revealed that the *S. mutans* luxS mutant forms altered biofilm structures compared by using dark-field microscopy to the wild-type strain on glass coverslips in BHI broth supplemented with 1% sucrose (34). Furthermore, these authors reported a noticeable difference in biofilm structure of the luxS mutant compared to that of the wild type based on visual inspection. Merritt et al. indicated that the luxS mutant had a very rough texture, and our present results confirm this at a higher magnification.

In contrast, biofilm formation by the previously attached luxS mutant cells was enhanced relative to the parental strain in the presence of sucrose. This result is compatible with our observation that the luxS mutant exhibited enhanced insoluble glucan synthesis and therefore autoaggregation relative to the strain GS-5 (data not shown). This would also lead to the attenuation of biofilm formation when the luxS mutant was initially inoculated in the presence of sucrose. The resulting large aggregates would not be able to attach and form biofilms as well as the parental strain. *S. mutans* synthesizes sucrose-derived glucans by Gtf enzymes (26) encoded by the *gtfB* (2, 48), *gtfC* (18), and *gtfD* (19) genes. Therefore, we analyzed *gtf* expression in relation to the luxS mutation. Real-time RT-PCR

**FIG. 6.** Complementation analysis of *S. mutans* luxS mutant by other oral streptococci. Bacteria were inoculated in the upper and lower wells in CDM supplemented with 0.5% sucrose for 48 h. Bacterial growth and biofilm formation of the *S. mutans* luxS mutant in the lower wells were measured. The data are the averages of three samples, and the standard errors are shown. Bars: ■, biofilm formation; □, bacterial growth. The CV-stained biofilms of the *S. mutans* luxS mutant complemented by other streptococci on polystyrene surface are shown above the graph. GS-5/GS-5, GS-5 complemented by GS-5 (positive control); ΔluxS, luxS mutant complemented by luxS mutant (negative control); GS-5, luxS mutant complemented by GS-5. *S. gordonii*, luxS mutant complemented by *S. gordonii* DL1; *S. oralis*, luxS mutant complemented by *S. oralis* ATCC 10557; *S. salivarius*, luxS mutant complemented by *S. salivarius* HT9R; *S. sanguinis*, luxS mutant complemented by *S. sanguinis* ATCC 10556; *S. sobrinus*, luxS mutant complemented by *S. sobrinus* MT8145; *S. anginosus*, luxS mutant complemented by *S. anginosus* FW73.

**FIG. 7.** Complementation analysis of *S. mutans* luxS mutant by periodontopathic bacteria. The *S. mutans* luxS mutant was inoculated into the diluted TSB medium (for *P. gingivalis*) or diluted THY medium (for *A. actinomycetemcomitans*) supplemented with 0.5% sucrose (34). Furthermore, these authors reported a noticeable difference in biofilm structure of the luxS mutant compared to that of the wild type based on visual inspection. Merritt et al. indicated that the luxS mutant had a very rough texture, and our present results confirm this at a higher magnification.

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in the present study revealed that gtfB and gtfC genes, coding for the Gtfs synthesizing insoluble glucans, were induced in mid-log phase compared to those of the parental strain. However, the gtfD gene coding for the Gtf synthesizing water-soluble glucans was not induced under these conditions. The gtfB/D/C transcript levels of both strains were almost the same in the early-log and stationary growth phases. Therefore, the luxS mutant would be expected to synthesize more adherent insoluble glucans than the wild-type strain during growth. The laboratory of S. D. Goodman indicated that there is increased Gtf activity in the luxS mutants (34). Our results confirm this at the transcriptional level. Fong et al. reported that A. actinomycetemcomitans AI-2 activity was maximal in early- and mid-log phases, which influences the expression of the A. actinomycetemcomitans leukotoxin. Leukotoxin levels increased severalfold in early-log-phase cells after exposure to conditioned medium from recombinant E. coli cultures expressing luxS (14). On the other hand, in Salmonella enterica serovar Typhimurium the expression of the luxS gene is controlled by environmental factors, and AI production and signaling activity increase during the mid-to-late-exponential-growth phases (50). Thus, the observation that gtfB and gtfC expression of the S. mutans luxS mutant was enhanced relative to the parental strain suggests that QS may also regulate insoluble glucan synthesis by S. mutans.

The widespread distribution of the luxS gene and the observation that AI-2 is capable of inducing a response in heterologous organisms suggests that the AI-2 signal system transcends species barriers and may function to signal the total bacterial cell community and/or influence its the metabolic potential (3, 51). The LuxS-based signaling system may be relevant for organisms in mixed oral biofilms such as dental plaque. Fong et al. (14) recently reported that A. actinomycetemcomitans AI-2 complemented a P. gingivalis luxS mutant (14). In the present study, we used a two-compartment system to examine complementation analysis of the S. mutans luxS mutant. We confirmed this system was useful for complementation analysis between two organisms. In addition, we examined whether bacterial culture supernatants restore biofilm formation of the luxS mutant by using the two-compartment assay system. We confirmed that bacterial supernatants restore biofilm formation by the luxS mutant using the two-compartment assay system (data not shown). Although this method is not as sensitive as the V. harveyi system (5), it is convenient and does not require a luminometer. Using the complementation assay system, biofilm formation by the S. mutans luxS mutant was restored to the same levels as the parental strain by S. gordonii, S. sobrinus, and S. anginosus strains. We confirmed the existence of the luxS gene in these streptococci by PCR, and all strains used in the present study possessed the luxS homologue (data not shown). Therefore, the noncomplementing streptococci may produce factors that antagonize AI-2 activities, and this is currently under investigation. We also confirmed the complementation of S. mutans luxS mutant biofilm formation by using the periodontopathic bacteria P. gingivalis and A. actinomycetemcomitans. However, the P. gingivalis luxS mutant did not restore biofilm formation by the S. mutans luxS mutant. Furthermore, we performed reporter assays with these oral bacteria and V. harveyi to confirm AI-2 production by the oral streptococci. The levels of AI-2 produced by oral streptococci and biofilm formation by the luxS mutant complemented by these bacteria were generally directly associated (Fig. 6 and Table 3). However, it is not clear why this was not the case with S. salivarius. This may suggest that additional factors are also expressed by some streptococci which also modulate AI-2 activity. These results provide additional support for the previous suggestion that LuxS-dependent intercellular signaling may modulate interspecies communication in oral biofilms. However, the functional significance of such interactions still remains to be determined.

### ACKNOWLEDGMENTS

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### REFERENCES


### TABLE 3. AI-2 bioassay of oral streptococci

<table>
<thead>
<tr>
<th>Organism with LuxS</th>
<th>AI-2 activity (% luminescence relative to V. harveyi BB1200* ± SD</th>
</tr>
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<tbody>
<tr>
<td>S. mutans GS-5</td>
<td>7.91 ± 1.82</td>
</tr>
<tr>
<td>S. mutans ΔluxS</td>
<td>0.00 ± 0.01</td>
</tr>
<tr>
<td>S. gordonii DLI</td>
<td>9.10 ± 1.50</td>
</tr>
<tr>
<td>S. oralis DL1</td>
<td>9.10 ± 0.77</td>
</tr>
<tr>
<td>S. salivarius MTH9R</td>
<td>16.71 ± 2.71</td>
</tr>
<tr>
<td>S. sobrinus MTH145</td>
<td>8.89 ± 2.41</td>
</tr>
<tr>
<td>S. anginosus FW73</td>
<td>14.11 ± 1.15</td>
</tr>
</tbody>
</table>

* n = 3.